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Gel Filtration Applied to the Study of Lipases and Other Esterases

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1. Sephadex G-100 and G-200 gel-filtration columns were calibrated for molecular-weight estimation with proteins of known molecular weights, and used to study the composition of several lipase or esterase preparations. 2. Enzymes from cow's milk, rat adipose tissue and pig pancreas were detected in the column effluents by their ability to liberate free acid from emulsified tributyrin at pH 8·5. 3. Four tributyrinases were detected in preparations from individual cow's milks. Molecular weights 62 000, 75 000 and 112 000 were estimated for three of them, but although the fourth may be of unusually low molecular weight an estimate was not possible. 4. Extracts of rat adipose tissue apparently contained six tributyrinases (molecular weights 39 000, 47 000, 55 000, 68 000, 75 000 and 200 000) but the relative amounts of these enzymes varied widely from rat to rat. 5. Tributyrinase activity in juice expressed from pig pancreatic tissue was due mainly to one enzyme (molecular weight 42 000). On the other hand, activity in extracts of acetone-dried pancreas was confined to material of molecular weight $> 10^6$, which may be an aggregated form of the lower-molecular-weight enzyme. 6. Activity in fractionated wheat-germ extracts was assayed with emulsified triacetin substrate, and was evidently due to one enzyme (molecular weight 51 000). 7. Some problems arising in the application of gel filtration to the study of lipase–esterase systems were indicated.

Experiments with various substrates and enzyme inhibitors have provided evidence for the presence of mixtures of lipases or other esterases in milk (Frankel & Tarassuk, 1957*a*,*b*; Forster, Bendixen & Montgomery, 1959; Forster, Montgomery & Montour, 1961), in blood plasma (Aldridge, 1953*a*,*b*, 1954) and in adipose tissue (Ashburn, Brown & Lynn, 1960), but the individual enzymes in such mixtures are difficult to recognize and characterize in the presence of one another because of their low, overlapping substrate specificities. Efficient fractionation methods which can be applied to crude preparations will clearly be helpful for the investigation of these enzymes, both in determining the complexity of mixtures and for isolating individual components. The value in this connexion of electrophoretic methods of separation is indicated by studies of tissue (Paul & Fottrell, 1961; Ecobichon & Kalow, 1961, 1964) and plasma (Augustinsson, 1958) esterases. We have investigated the separation of lipases and esterases by gel filtration (Porath, 1962) in the expectation that the various enzymes differ from one another in molecular weight.

Good correlation exists between the gel-filtration behaviour and the molecular weights of many enzymes and other globular proteins (Andrews, 1962, 1964; Andrews, Bray, Edwards & Shooter, 1964; Wieland, Duesberg & Determan, 1963; Whitaker, 1963), so, besides being a means for separating proteins, gel filtration may enable their molecular weights to be estimated. However, as lipases or other esterases may differ from typical globular proteins in the correlation between molecular weight and gel-filtration behaviour, molecular weights now estimated for them are necessarily tentative. On the other hand the values may be useful for characterization because they do describe the behaviour of the enzymes in particular circumstances.

Lipases form a rather indefinite section of the esterase group of enzymes (cf. Desnuelle & Savary, 1963), and it is useful to distinguish lipases from other esterases by a modification of the definition used by Aldridge (1954) and Desnuelle (1961), namely that lipases hydrolyse emulsified substrates whereas other esterases hydrolyse dissolved substrates. A general method for assaying enzymes of both groups requires as substrate an ester which when emulsified exists partly in solution and partly in the dispersed phase, and which is hydrolysed by many enzymes of both groups. Tributyrin (glycerol tributyrate) seems to be suitable for the purpose (Aldridge, 1954; Desnuelle & Savary, 1963) and accordingly we have used tributyrin emulsions in most of our experiments. As the enzymes so

detected may be either lipase or other esterase, according to the definition, we call them tributyrinases until their nature is determined. However, the properties of the pancreatic enzyme are such (Desnuelle, 1961) that it may be designated a lipase. The wheat-germ enzyme hydrolyses tributyrin so slowly that triacetin (glycerol triacetate) was used instead for its assay, and its properties are those of an esterase (Mounter & Mounter, 1962).

Some of our gel-filtration experiments have been reported briefly (Downey & Andrews, 1964).

MATERIALS AND METHODS

Cow's-milk tributyrinases. Tributyrinase activity in cow's milk is associated mainly with the casein micelles, but solutions were obtained containing about 70% of the total tributyrinase in each milk from a number of individual Friesian cows by adding NaCl (0·75 M final concn.) to the skim milks and sedimenting most of the undissolved casein by centrifugation in a Spinco model L ultracentrifuge (rotor 30) at 80000 *g* for 1 hr.

Rat adipose-tissue tributyrinases. Separate extracts were prepared from retroperitoneal adipose tissue from each of seven freshly killed female hooded Norway rats by homogenizing the tissues in equal volumes of ice-cold 0·025 M-$MgCl_2$, then centrifuging the mixtures at 10000*g* for 15 min. and separating the clear solutions.

Pig pancreatic lipase. Two preparations were examined: (A) an extract of acetone-dried powder of pig pancreas (L. Light and Co. Ltd., Colnbrook, Bucks.) with five times its weight of 0·75 M-NaCl containing 0·025 M-$MgCl_2$, which had been clarified by centrifugation at 2000 *g* for 10 min. and then diluted 20-fold with the extraction medium; (B) the juice expressed from fresh pig pancreatic tissue by compressing it in a perforated stainless-steel cup with a stainless-steel plug and a hydraulic press was diluted 1000-fold with 0·75 M-NaCl containing 0·025 M-$MgCl_2$.

Wheat-germ esterase. A freeze-dried preparation of wheat-germ esterase (L. Light and Co. Ltd.) was dissolved (10 mg./ml.) in 0·75 M-NaCl containing 0·025 M-$MgCl_2$.

Enzyme substrates. Substrate for assay of milk and adipose-tissue tributyrinases and pancreatic lipase was a freshly prepared tributyrin emulsion, obtained by adding tributyrin (British Drug Houses Ltd., Poole, Dorset) (10 ml.) to gum acacia (British Drug Houses Ltd.) (10 g.) and ice–water (70 ml.) which had already been blended in a rotating-blade homogenizer (Ato-Mix; Measuring and Scientific Equipment Ltd., London) and homogenizing the mixture for 5 min. at full speed. The pH of the resultant emulsion was adjusted to 8·5 with N-NaOH, and water added to a final volume of 100 ml. Wheat-germ esterase was assayed with a similarly prepared emulsion containing triacetin (British Drug Houses Ltd.) (20 ml.) instead of tributyrin, with the pH adjusted to 7·5.

Assay of lipase and esterase activity. Activity was measured by continuously titrating with 1 mN-NaOH the acid liberated from the appropriate substrate, by using a Radiometer titrator type TTT1c, coupled to a Radiometer titrigraph type SBR2c (Radiometer, Copenhagen, Denmark). The reaction occurred in a water-jacketed vessel (inner dimensions 2·3 cm. diam. × 7·7 cm. deep) containing 2–8 ml. of assay mixture, and which had a stoppered side arm near the

Table 1. *Proteins used to calibrate Sephadex gel-filtration columns for estimation of protein molecular weight*

Protein	Mol. wt. (reference)	Amount used (mg.)	Method of estimation	Column calibrated G-100	Column calibrated G-200
Glucagon	3500 (Bromer *et al.* 1957)	1–1·5	*E* (230 mμ)	+	
Cytochrome *c* (from horse heart)	12400 (Margoliash, 1962)	1–3	*E* (412 mμ)	+	+
Chymotrypsinogen	25000 (Wilcox, Krant, Wade & Neurath, 1957)	1·5 or 4	*E* (230 or 280 mμ)	+	+
Adenosine deaminase	34000 (Andrews, 1964)	0·01	Disappearance of adenosine followed at 265 mμ (Kaplan, 1955)	+	
Serum albumin (bovine)	67000 (Phelps & Putnam, 1960)	7–10	*E* (280 mμ)	+	+
Phosphatase (from *Escherichia coli*)	78000 (Garen & Levinthal, 1960)	0·05	Hydrolysis of *p*-nitrophenyl phosphate at pH 8·0 (Garen & Levinthal, 1960)	+	
Lactate dehydrogenase (from rabbit muscle)	135000 (Fromm, 1963; Pesce, Stolzenbach, Freedberg & Kaplan, 1963)	0·05	Oxidation of NADH (Kornberg, 1955)	+	
γ-Globulins (human)	Effectively 205000 in gel filtration when compared with globular proteins containing no carbohydrate (P. Andrews, unpublished work)	3–5	*E* (280 mμ)	+	
Xanthine oxidase	275000 (Andrews *et al.* 1964)	~1	Oxidation of xanthine followed at 295 mμ (Andrews *et al.* 1964)	+	
Apoferritin	480000 (Harrison, 1963)	~1	*E* (280 mμ)	+	+
β-Galactosidase (from *E. coli*)	520000 (Sund & Weber, 1963)	0·1	Hydrolysis of *o*-nitrophenyl β-D-galactopyranoside (Wallenfels, 1962)		

top. The assay mixture was continuously stirred and bubbled with CO_2-free N_2 [250 ml./min., measured with a Rotameter flow-meter (Rotameter Manufacturing Co. Ltd., Croydon, Surrey)]. Provided that the glass electrode had not lost its normal rapid response to small pH changes, the method satisfactorily measured rates of acid liberation up to 70 mμequiv./min. but addition of alkali by the titrator was not rapid enough to keep pace with rates of acid liberation greater than this. Glass electrodes type 202B (Radiometer) and type 11128 (W. G. Pye and Co. Ltd., Cambridge) both performed well for 6–8 weeks in assays with emulsified substrates, which seemed to be the main cause of electrode contamination, but thereafter gave increasingly erratic results, with high variable blank values.

In performing assays, enzyme solution was added first to the reaction cell, so that subsequent addition of substrate (tributyrin emulsion, 2·0 ml., or triacetin emulsion, 1·0 ml.) and water (if necessary to bring the total volume to 2 ml.) served to ensure complete transference of the enzyme to the region around the electrodes. Assays with tributyrin as substrate were carried out at pH 8·5, and those with triacetin at pH 7·5, but the enzyme–substrate mixtures were first adjusted to pH 8·7 and 7·7 respectively with alkali from the micro-syringe of the titrator, and recording of the titration was not commenced until the pH of the mixtures had dropped to the pre-set values. Rates of acid production recorded in this way were linear with time, and proportional to the amounts of enzyme added. Whenever possible, amounts of enzyme were chosen to give rates of acid liberation in the range 20–60 mμequiv./min., provided that the final assay volume was not more than three times the volume of emulsion used when tributyrin was substrate, or more than twice the volume of triacetin substrate. The reason for this precaution was to avoid complete dissolution of the emulsified substrate by excessive dilution.

Gel filtration. Sephadex G-100 gel-filtration medium (140–400 mesh; Pharmacia, Uppsala, Sweden) was allowed to swell for 7 days in 0·025 M-$MgCl_2$ before use, and G-200 (140–200 mesh) for 1–3 months in 0·75 M-NaCl containing 0·025 M-$MgCl_2$. Swelling of Sephadex G-200 in salt solutions proceeds rapidly for several hours, then continues much more slowly for many weeks, and even the slow swelling has a considerable effect on the upper exclusion limit of the gel (P. Andrews, unpublished work). Columns (50 cm. × 2·4 cm. diam.) were packed in the cold with the swollen gels as described by Flodin (1961) and equilibrated with salt solutions as indicated for the various experiments. Samples were applied to the tops of the columns by layering under the buffer already present, sucrose being added to the samples to increase their density if required. Flow rates were about 30 ml./hr. for the G-100 columns and about 15 ml./hr. for the G-200 ones. Effluent was collected in 3 ml. (G-100 experiments) or 5 ml. (G-200) fractions with a collector (Aimer Products Ltd., London) fitted with a siphon. All columns were run at 0–5°.

The columns were calibrated for estimating the molecular weights of proteins in the manner described by Andrews (1964), by using as standards the proteins of known molecular weights listed in Table 1. Of these, crystalline or highly purified samples of glucagon, adenosine deaminase, xanthine oxidase, apoferritin and β-galactosidase were generously provided by Dr W. W. Bromer (Eli Lilly and Co.), Professor T. G. Brady, Dr R. C. Bray, Dr Pauline M. Harrison and Professor K. Wallenfels respectively. The other proteins were commercial crystalline or highly purified grades. The calibration graphs (V_e against log mol.wt.) for the Sephadex G-100 and G-200 columns were respectively similar to those given by Andrews (1964) and Andrews *et al.* (1964) for other columns of the same gels, with extensions of the Sephadex G-200 graphs towards lower molecular weights resembling in shape the G-100 graphs in the same region.

It may be noted that although ovalbumin (mol.wt. 45000) is often useful as a molecular-weight standard, it proved unsuitable in the experiments described here because when dissolved in solutions containing Mg^{2+} ions it was eluted from gel-filtration columns earlier (apparent mol.wt. 54000) than in the absence of Mg^{2+}.

The enzymes listed in Table 1 were included in some column runs with tributyrinase preparations to act as internal molecular-weight standards. The quantities used were such as to permit enzyme assays on small portions (0·1–0·3 ml.) of the effluent fractions. Cytochrome *c* (2 mg.) was often added as a standard, and to check for even flow in the columns. None of the additions had a detectable effect on the gel-filtration behaviour of the tributyrinases. Elution volumes of the tributyrinases and other proteins in the tributyrinase preparations were interpreted in terms of molecular weights by use of the calibration curves for the columns.

Lactose from the milk-tributyrinase preparations was estimated in column effluents by an anthrone method (Trevelyan & Harrison, 1952).

RESULTS

Cow's-milk tributyrinases. Milks obtained from different cows during October and November 1963 differed from one another in tributyrinase activity over a threefold range. Five of the more active ones gave soluble preparations of which 1 ml. portions liberated 0·7–1·0 μequiv. of acid/min. from the tributyrin emulsion, and these were used (2–4 ml. portions) for gel filtration through a Sephadex G-200 column. The column eluent contained 0·75 M-sodium chloride to prevent readsorption of tributyrinases to the casein micelles still present in the preparations, and 0·025 M-magnesium chloride because dialysis experiments have indicated that part of the tributyrinase activity in the preparations is dependent on the presence of Mg^{2+} or certain other bivalent cations (W. K. Downey, unpublished work).

A typical elution diagram for these preparations (Fig. 1*a*) shows a peak representing low-molecular-weight material, some of which is retarded in the column rather more than is lactose (V_e 212 ml.), and four other peaks which from their elution volumes and the corresponding molecular weights may be ascribed to milk proteins as indicated in Table 2.

Figs. 1*b–d* are elution diagrams for tributyrinase in preparations from three different milks. The diagrams for individual milks were reproducible, and milks taken from the same cow at different times during the 2-month sampling period showed

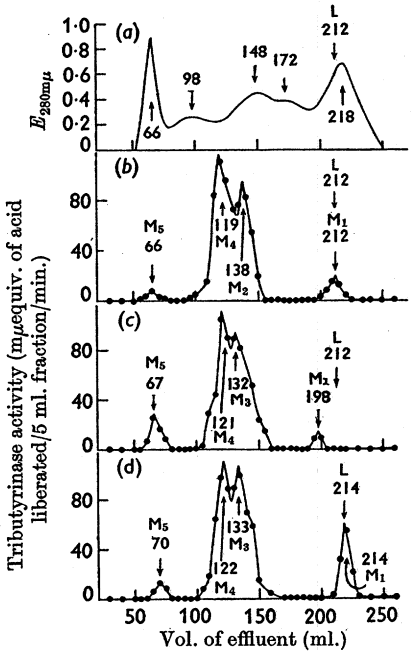

Fig. 1. Gel filtration of preparations of cow's-milk tributyrinase on a Sephadex G-200 column. Tributyrinase assays were performed on 1–4 ml. portions of column effluent. (*a*) Material absorbing at 280 mμ; (*b*)–(*d*) tributyrinase activity in preparations from different milks. M_1–M_5 and L are designations for tributyrinase and lactose peaks respectively, and the numbers are their elution volumes. See the text for details.

no significant variation. On the other hand, variation between different milks is evident. Tributyrinase activity in the effluent fractions decreased steadily, even during storage in the cold, and very little remained after 3 days. When the fractions were assayed within 8 hr. of collection, the recovery of tributyrinase, compared with that applied to the column, was about 40%. Molecular weights for the tributyrinases, derived from their gel-filtration behaviour, are listed in Table 2.

Rat adipose-tissue tributyrinases. Enzyme preparations from different rats differed considerably in tributyrinase activity (0·3–4·5 μequiv. of acid liberated from tributyrin emulsion substrate/ml. of preparation/min.). Samples used for gel-filtration experiments were of different sizes (2 or 4 ml.), depending on their activity. Preliminary experiments with Sephadex G-200 indicated that none of the tributyrinases had molecular weights above 200000; therefore subsequent experiments utilized the better resolving power of G-100 for lower-molecular-weight proteins.

Magnesium chloride (0·025 M) was initially chosen as column eluent to avoid possible inhibition of tributyrinase by sodium chloride (Willart & Sjöström, 1959). Some runs were repeated with 0·2 M-sodium chloride added to the eluent to check for adsorption of enzyme to the gel at the lower ionic strength, but no significant differences were observed in the results.

Judging from a typical elution diagram (Fig. 2*a*), the enzyme preparations contained only small

Table 2. *Interpretation of gel-filtration elution diagrams of tributyrinase preparations from cow's milk*

The elution diagrams are shown in Fig. 1. Molecular weights were derived from the elution volumes (V_e) by use of the calibration graph of the column (Sephadex G-200). The graph indicated a minimum molecular weight of at least 10^6 for proteins excluded from the gel pores (V_e 66 ml.). See the text for further discussion of the results.

Elution diagram	V_e of peak (ml.)	Mol.wt. (±10%) corresponding to V_e	Suggested identity of material represented by peak
Fig. 1(*a*)	66	$>10^6$	Casein micelles
	98	220000	Casein aggregates (heterogeneous)+milk globulins (mol.wts. ~ 180000*)
	148	36000	β-Lactoglobulin (36500)†
	172	19000	α-Lactalbumin (15500) or perhaps α-lactalbumin admixed with casein monomers (20000–27000)
	218	<3000	Complex mixture of low-molecular-weight material
Figs. 1(*b*)–(*d*)	66, 67, 70	$>10^6$	Tributyrinase(s) associated with casein micelles
	119, 121, 122	112000	Tributyrinase M_4
	132, 133	75000	Tributyrinase M_3
	138	62000	Tributyrinase M_2
	198, 212, 214	See the text	Tributyrinase M_1

* As glycoproteins these globulins probably behave on gel filtration as ~ 200000 (cf. γ-globulins, Table 1).
† At concentration high enough to suppress its dissociation (cf. Andrews, 1964).

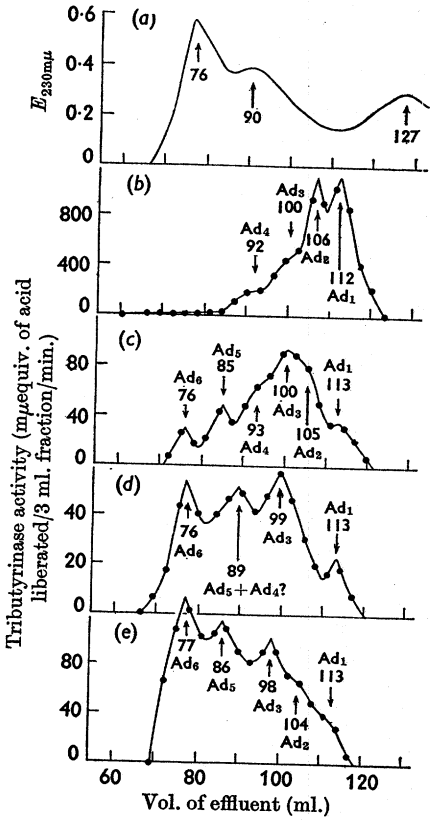

Fig. 2. Gel filtration of tributyrinase preparations from rat adipose tissue on a Sephadex G-100 column. Tributyrinase assays were performed on 0·2–2 ml. portions of column effluent. (*a*) Material absorbing at 230 mμ; (*b*)–(*e*) tributyrinase activity in different preparations. Ad_1–Ad_6 and the numbers indicate proposed individual enzymes and their elution volumes respectively. See the text for details.

amounts of protein. A faint pink colour in the column effluent coinciding with the peak at 127 ml. indicated that it represented haemoglobin; therefore the other two peaks may also be due, at least in part, to traces of other blood proteins (Table 3). A peak for low-molecular-weight material was not detected.

Elution diagrams for tributyrinase in four of the enzyme preparations (Figs. 2*b*–*e*) show the wide variation that was observed, undoubtedly more than might be accounted for by incomplete recoveries ($\simeq$ 70%) of tributyrinase applied to the column. Three other enzyme preparations gave diagrams resembling Figs. 2(*b*) or 2(*c*), rather than 2(*d*) or 2(*e*). Tributyrinase activity in the effluent fractions decreased during storage in the cold, but more slowly than with the milk enzymes. Molecular weights for the adipose-tissue tributyrinases, calculated from their gel-filtration behaviour, are given in Table 3.

Pig pancreatic lipase. Enzymic activity/ml. in preparation A liberated 155 μequiv. of acid/min. and that in preparation B liberated 16·5 μequiv./min. from the tributyrin substrate; hence both preparations were much more active than those from milk or from adipose tissue. Gel filtration was performed with 0·2 ml. portions of A diluted to 2 ml. with column eluent or 2·0 ml. portions of B on Sephadex G-200 columns, equilibrated for some experiments with 0·75 M-sodium chloride containing 0·025 M-magnesium chloride, and for others with 0·1 M-sodium chloride only. Identical results were obtained under both conditions.

The elution diagram of preparation A (Fig. 3*a*) shows a very small peak at the void volume of the column (72 ml.) due to protein excluded from the gel pores, and a relatively much larger one (V_e 214 ml.) probably representing low-molecular-weight material. All the lipase detected in the column effluent (75% recovery) formed a peak at the void volume, indicating for it a molecular weight $> 10^6$.

The very small amounts of protein in preparation B gave peaks (Fig. 3*b*) at the void volume of the column (68 ml., mol.wt. $> 10^6$), at 110 ml. (mol.wt. 180000) and at 162 ml. (mol.wt. 37000), but some of it was not resolved into peaks. A peak for low-molecular-weight material was not detected. The recovery of lipase was about 50% of that applied to the column. Of this, 3% was eluted in a peak at the void volume (mol.wt. $> 10^6$), whereas the remainder formed a peak with V_e 158 ml. (mol.wt. 42000) which did not coincide with any of the protein peaks.

Wheat-germ esterase. Esterase activity/ml. of the preparation liberated 0·5 μequiv. of acid/min. from the triacetin substrate, and 2 ml. portions were used for gel filtration on a Sephadex G-100 column, equilibrated either with 0·75 M-sodium chloride plus 0·025 M-magnesium chloride or with 0·2 M-sodium chloride. Each experiment gave the same result. Protein in the preparation emerged from the column both at the void volume (74 ml.) and distributed throughout later fractions (Fig. 4). Esterase activity was quantitatively recovered in a single peak (V_e 104 ml., mol.wt. 51000), which showed no obvious signs of heterogeneity.

DISCUSSION

The gel-filtration experiments demonstrate different degrees of complexity for lipase or esterase preparations from various sources, and in this respect the results are in accord with current ideas on the nature of the mixtures examined. The wheat-germ preparation is evidently the simplest of them, as might be predicted from the work of

Table 3. *Interpretation of gel-filtration elution diagrams of tributyrinase preparations from rat adipose tissue*

The elution diagrams are shown in Fig. 2. Molecular weights were derived from the elution volumes (V_e) by use of the calibration graph of the column (Sephadex G-100). Material excluded from the gel pores was eluted from the column at 74 ml., hence neither the protein (V_e 76 ml.) nor the tributyrinase (V_e 76, 77 ml.), which coincided in position with γ-globulins (apparent mol.wt. ~ 200000; see Table 1), passed through the column unretarded. See the text for further discussion of the results.

Elution diagram	V_e of peak (ml.)	Mol.wt. (±10%) corresponding to V_e	Suggested identity of material represented by peak
Fig. 2(*a*)	76	200000	γ-Globulins + other proteins?
	90	75000	Serum albumin + other proteins?
	127	27000	Haemoglobin at sufficiently low concn. for it to be partially dissociated into sub-units during gel filtration (Andrews, 1964)
Figs. 2(*b*)–(*e*)	76, 77	200000	Tributyrinase Ad_6
	85, 86	94000	Tributyrinase Ad_5
	89	75000	Tributyrinases (Ad_4 + Ad_5?)
	92, 93	68000	Tributyrinase Ad_4
	98–100	55000	Tributyrinase Ad_3
	104–106	47000	Tributyrinase Ad_2
	112, 113	39000	Tributyrinase Ad_1

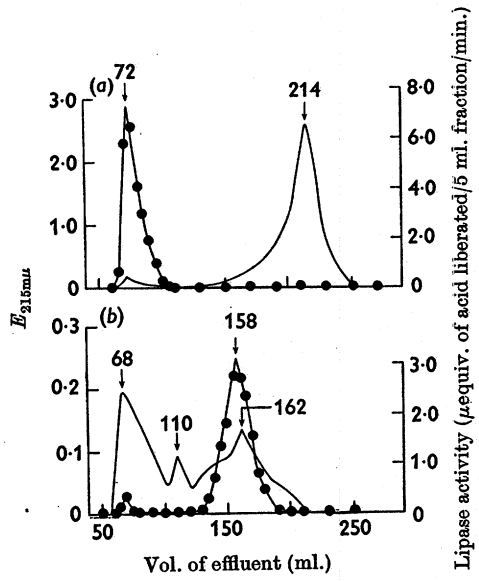

Fig. 3. Gel filtration of pig pancreatic lipase preparations A (*a*) and B (*b*) on different Sephadex G-200 columns. Lipase assays were performed on 0·1 or 0·2 ml. portions of column effluent for the major peaks and on 4 ml. portions otherwise. ——, Extinction at 215 mμ; ●–●, lipase activity. The numbers are elution volumes of the indicated peaks. See the text for details.

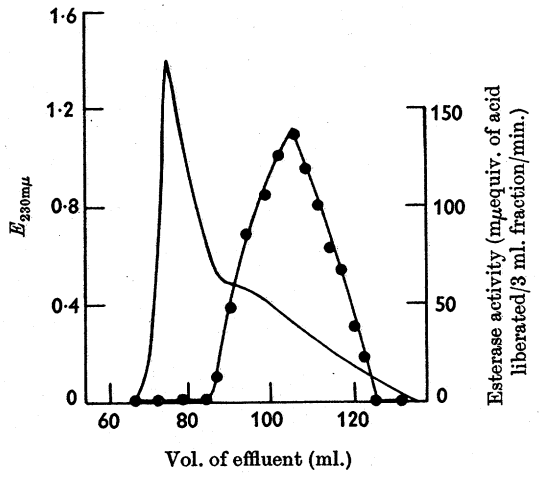

Fig. 4. Gel filtration of a preparation of wheat-germ esterase on a Sephadex G-100 column (see text for details). Esterase assays were performed on 1·0 ml. portions of effluent. ——, Extinction at 230 mμ; ●—●, esterase activity.

Mounter & Mounter (1962). Gel filtration indicates that its hydrolytic activity towards triacetin is due to a single enzyme with molecular weight about 51000. Although assays on the column effluents were performed with emulsified substrate in a search for separate lipase activity, none was detected. Judging from Fig. 3*b*, hydrolysis of tributyrin by pig pancreatic juice is also effected mainly, if not entirely, by one enzyme, in this case of molecular weight about 42000, and this presumably is the pancreatic lipase studied extensively by Desnuelle and his co-workers (Desnuelle, 1961). The absence of a corresponding peak for enzymic activity from the elution diagram of acetone-dried pancreatic tissue extract (Fig. 3*a*) is unexpected. Activity in these extracts is associated instead with

very high-molecular-weight material, of which a small amount is also present in the juice, possibly due to contamination of the juice with cellular contents. The existence of different lipases with widely differing molecular weights in the two preparations seems unlikely. Instead, the two preparations may contain different forms of the same enzyme, the free form existing in the juice, and a bound or aggregated form appearing in the tissue extract. The presence of so little protein relative to enzymic activity in column fractions containing the high-molecular-weight form of the enzyme (Fig. 3*a*) argues against the possibility that this form consists of the rather small enzyme protein molecules associated with much larger inactive proteins, and indicates instead that it is a highly aggregated form of the enzyme. However, no aggregates of intermediate size were detected. Gelotte (1964) finds that high- and low-molecular-weight forms of lipase can be extracted from acetone-dried pancreatic tissue in relative amounts which depend on the extraction conditions, but it is uncertain whether the differences in relative amount are determined by the accessibility or solubilities of the two forms, or whether an interconversion between them is involved. The conclusion (Desnuelle, 1961) that pancreatic extracts contain only one enzyme capable of hydrolysing tributyrin may yet prove to be correct. Gelotte's estimate of a molecular weight 35000 for the low-molecular-weight form from its gel-filtration behaviour agrees reasonably well with our own figure.

Tributyrinase activity in cow's milk is evidently due to several enzymes (Fig. 1) of different molecular weights (Table 2) which vary in relative amounts in different milks. Although the enzymes were not all separated one from another sufficiently to give distinct peaks in the elution diagrams, a comparison of several diagrams (Figs. 1*b–d*), plus the reproducibility in gel filtration of elution volumes for individual proteins, indicates that much of the tributyrinase activity in milk is due to three enzymes (M_2, M_3 and M_4). The lipase of molecular weight 7000 isolated from milk clarifier slime (Chandan & Shahani, 1963; Chandan, Shahani, Hill & Scholz, 1963) could be represented by the peaks labelled M_1. The position of M_1 in Fig. 1(*b*) corresponds to a molecular weight only 4000, but the variations which occur in its elution volume, which are easily detected by comparing its position with that of lactose (L), indicate that it was adsorbed to the gel, the adsorption being influenced possibly by small changes in temperature or eluent flow rate. As M_1 gave satisfactory peaks in the elution diagrams its adsorption may have been weak and the retardation on its passage through the column quite small, hence its molecular weight may not be much greater than 4000.

The possibility does exist that the peaks M_1, M_2, M_3 and M_4 are due to one enzyme of low molecular weight existing both in the free form (M_1) and in association with casein or other proteins. Calculations based on a molecular weight 7000 for the free enzyme, molecular weights 20000–27000 for the various casein monomers (McKenzie & Wake, 1959) and the molecular weights given in Table 2 for M_2, M_3 and M_4 indicate that, if the latter do consist of the low-molecular-weight enzyme associated with casein, then dimeric to pentameric forms of casein should be involved. The tendency of casein molecules to aggregate is strong and the formation of these limited aggregates seems unlikely. On the other hand, it does seem likely that the peaks labelled M_5 represent one or more of the other enzymes still attached to highly aggregated forms of casein, even though much of the enzyme originally associated with the casein micelles has been removed by addition of sodium chloride to the milk.

Judging from the diagrams in Figs. 1 and 2, the mixtures of tributyrinases which occur in rat adipose tissue are more complex and more variable than those in cow's milk, although in so far as traces of haemoglobin were detected in the adipose-tissue preparations, and rat blood contains tributyrinase (Aldridge, 1954; Augustinsson, 1958), some of the complexity in the adipose-tissue preparations might be due to serum enzymes. Although tributyrinase activity in one adipose-tissue preparation (Fig. 2*b*) was evidently due to four enzymes, of which two (Ad_1 and Ad_2) were predominant, other preparations (Figs. 2*c–e*) were more complex, with different enzymes predominating. Clearly, incomplete resolution results in uncertainties about the number of enzymes present in such mixtures. The broad peak at 89 ml. in Fig. 2(*d*) was not present in other diagrams and is tentatively attributed to a mixture of enzymes. Possibly it represents roughly equal amounts of Ad_4 and Ad_5, since it occurs midway between their two positions, although Fig. 2(*c*) shows that, when the relative amounts of Ad_4 and Ad_5 are favourable, they are separated from each other. The peaks at 76–77 ml. in Figs. 2(*c*)–(*e*) are near, but not at, the void volume of the column, so they could be due to mixtures of quite high-molecular-weight enzymes or, less likely, because the molecular weights would not be far above 200000, to highly aggregated or bound forms of enzyme. The peaks are attributed to one tributyrinase (Ad_6) because gel filtration on Sephadex G-200 of a preparation similar to the one shown in 2(*c*) indicated the presence therein of only one tributyrinase of molecular weight about 200000. By attributing peaks or pronounced inflexions which recur at similar places in more than one elution diagram to individual enzymes, we conclude that rat adipose-tissue extracts may contain as

many as six tributyrinases, and perhaps even more. One of them may well be analogous to the tributyrinase isolated from pig adipose tissue by Lynn & Perryman (1960).

Our experiments illustrate some of the problems which may arise in applying gel filtration to lipase–esterase systems. In studying crude mixtures it may be difficult, as with some milk-tributyrinase preparations, to apply sufficient enzyme to a gel-filtration column for enzyme assays on the column effluent to be accomplished satisfactorily. The ability of lipases to attack emulsified substrates suggests that they may have adsorptive properties which could retard their passage through a column, as with milk-tributyrinase M_1, or cause low recoveries of enzyme from a column. Serious losses of pancreatic lipase during chromatography can be obviated by using comparatively large amounts of enzyme (Professor P. Desnuelle, personal communication). Low recoveries of the milk tributyrinases seem to be due in some measure at least to their instability when detached from the casein micelles, and the instability may be enhanced by a high sodium chloride concentration in the column eluent (Willart & Sjöström, 1959). Surprisingly enough, their instability may have been advantageous, for we attribute the narrowness of the milk-tributyrinase peaks, resulting in unusually good resolution of the middle ones (Fig. 2), to instability of the enzymes, with the greatest losses of activity occurring in the effluent fractions in which the enzymes were at lowest concentration.

Certain bivalent ions may be necessary for the activity of lipases of milk (see above) and other sources (Desnuelle, Naudet & Constantin, 1951; Wood, 1959; Stansfield, 1960), and for this reason we included magnesium chloride in our enzyme preparations and in the column eluents, but subsequent experiments indicate that its omission has little, if any, effect on the results, nor has its inclusion in the substrate emulsions. The pH optima of lipases are generally about pH 8–9, and our tributyrinase assay was performed at pH 8·5 to conform with this. Nevertheless, our methods are unlikely to detect all the lipases or esterases present in complex mixtures. Forster *et al.* (1959, 1961) have shown that the lipase–esterase activity of milk is probably due to several enzymes or groups of enzymes, some of which have practically no activity towards tributyrin, and from the work of Ashburn *et al.* (1960) the same is true of adipose tissue. Again, the tributyrinase assay did not fulfil the specific requirements for reactivity of the lipoprotein lipase of either source (Korn, 1959, 1962*a*,*b*). Despite the uncertainties, gel filtration still seems a method of promise for the study of soluble lipase–esterase systems, even ones as intractable as that occurring in milk.

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